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• LIVER CANCER •

Mutation analysis of novel human liver-related putative tumor suppressor gene in hepatocellular carcinoma

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Abstract

AIM: To find the point mutations meaningful for inactivation of liver-related putative tumor suppressor gene (LPTS) gene, a human novel liver-related putative tumor suppressor gene and telomerase inhibitor in hepatocellular carcinoma.

METHODS: The entire coding sequence of LPTS gene was examined for mutations by single strand conformation polymorphism (SSCP) assay and PCR products direct sequencing in 56 liver cancer cell lines, 7 ovarian cancer and 7 head & neck tumor cell lines and 70 pairs of HCC tissues samples. The cDNA fragment coding for the most frequent mutant protein was subcloned into GST fusion expression vector. The product was expressed in *E.coli* and purified by glutathione-agarose column. Telomeric repeat amplification protocol (TRAP) assays were performed to study the effect of point mutation to telomerase inhibitory activity.

RESULTS: SSCP gels showed the abnormal shifting bands and DNA sequencing found that there were 5 different mutations and/or polymorphisms in 12 tumor cell lines located at exon2, exon5 and exon7. The main alterations were A(778)A/G and A(880)T in exon7. The change in site of 778 could not be found in HCC tissue samples, while the mutation in position 880 was seen in 7 (10 %) cases. The mutation in the site of 880 had no effect on telomerase inhibitory activity.

CONCLUSION: Alterations identified in this study are polymorphisms of LPTS gene. LPTS mutations occur in HCC but are infrequent and of little effect on the telomerase inhibitory function of the protein. Epigenetics, such as methylation, acetylation, may play the key role in inactivation of LPTS.

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INTRODUCTION

Human hepatocellular carcinoma (HCC), the predominant histological subtype of primary liver cancer is one of the most common malignancies of the liver worldwide. The development of human cancer results from the clonal expansion of genetically modified cells that acquired selective growth advantage through accumulated alterations of proto-oncogenes and tumor suppressor genes[1]. Chromosomal analysis using polymorphic DNA markers that distinguish different alleles has revealed loss of heterozygosity (LOH) of specific chromosomal regions in various types of cancers and mapping of regions with a high frequency of LOH has been critical for identifying negative regulators of tumor growth, which will be of great help in positional cloning of tumor suppressor genes[2].

We have cloned a novel human liver-related putative tumor suppressor gene, LPTS by means of allelic-loss mapping and positional candidate cloning[3]. LPTS gene mapped to chromosome 8p23, a locus with high-frequency LOH and a hot spot of tumor suppressor in HCC[2, 4]. The expression of LPTS was ubiquitous in normal human tissues, whereas levels appeared to be significantly reduced, or sometimes undetectable in HCC cells and neoplastic tissues. The gene for LPTS is a growth-arrest gene that acts directly or indirectly to control the proliferation of cells, and might be a tumor suppressor gene[3]. LPTS gene has 7 exons totally and encodes two transcripts, one is LPTS-S, lacking exon6 and encoding a 174-a.a. protein; the other is LPTS-L, encoding a 328-a.a. protein with entire 7 exons. The LPTS-L is highly homologous to PinX1 identified recently[5]. LPTS-L and PinX1 have quite different 3' -untranslated region and encode a same protein, referred to LPTS-L/PinX1. LPTS-L/PinX1 has strong telomerase inhibitory activity both *in vivo* and *in vitro*.

In this study, we collected 56 HCC cell lines, 7 ovarian cancer cell lines and 7 head & neck tumor cell lines to perform single strand conformation polymorphism (SSCP) assay[6,7] to screen the point mutations in LPTS gene. Then we detected those mutations identified from SSCP assay in 70 pairs of HCC tissues to confirm the existence of real mutations in HCC tissue samples. Finally, telomerase activity assay was done to study the effect of the point mutation on the function of protein *in vitro*.

MATERIALS AND METHODS

Tumor cells and HCC tissue samples

The cell lines, including 56 liver cancer cell lines, 7 ovarian cell lines and 7 head and neck tumor cell lines, were from mainland of P.R. China, Japan, France, Taiwan, Hongkong and America separately. The cell lines were cultured in Dulbecco' s Modified Eagle Medium (DMEM, Life Technologies Inc., Grand Island, NY) or in RPMI medium 1 640 (Life Technologies Inc.),

supplemented with 10 % fetal calf serum (FCS, Life Technologies Inc.) with or without non-essential amino acid (Life Technologies Inc.) according to suppliers. All the samples of primary HCC (Ks), adjacent samples of nontumorous tissues (Ls), were obtained from Shanghai Eastern Hepatobiliary Surgery Hospital (Shanghai, P.R. China), with the agreement of each patient. The serial numbers were those recorded by the hospital. All tissues were placed in liquid nitrogen immediately after surgical resection.

Point mutation assay

According to the genome sequence of LPTS gene, we synthesized 11 pairs of primers covering 7 exons of the gene, with length of each PCR product around 170 to 250 bp (Table 1). Genomic DNA was separated as described[8]. PCR was performed in a 25-μL reaction mixture that contained 2 μL Genomic DNA (around 50 ng) from each cell line as template, 2 μL primers mix (20 μM each), 0.4 μL dNTP mix (12.5 mM each), 4.75 μL H_2O, 0.25 μL Taq DNA polymerase (Life Technologies Inc.), and 15.6 μL premix (16 mM TrisHCl pH8.4, 80 mM KCl, 2.4mM $MgCl_2$ and 0.16 % Tween-20). PCR reaction conditions were described in Table 1. In Table 1, SD referred to the step-down PCR, the annealing temperature was declined 3 ℃ every 3 cycles for 94 ℃ 45 sec, annealing temperature 1 min and 72 ℃ 1 min from 68 ℃ until the indicated temperature and then for another 25 cycles of standard PCR reaction. The products of PCR were separated by electrophoresis on 2 % agarose gel to check the specificity of each PCR reaction. The PCR products were sequenced directly or performed SSCP assay as described below.

Table 1 Primer sequences for the coding region of the LPTS gene

Exon	Nucleotide sequences	PCR conditions	Product size (bp)
1	Forward: 5’ -CGTGCTCGAGGAGCGAGTCG-3’		
	Reverse: 5’ -ACCCGGCATCTTCACCAACG-3’	52 ℃ SD	241
2	Forward: 5’ -TCCATTGCTGATGATAATGC-3’		
	Reverse: 5’ -CTTCCAGTCTCCTAAGAAGG-3’	50 ℃ SD	230
3	Forward: 5’ -TGAGAGGAATGTTCTAACTC-3’		
	Reverse: 5’ -AGCCAAYYAYGCAAAGACAC-3’	50 ℃ SD	178
4	Forward: 5’ -AACTACAGGCTTACCTCTCG-3’		
	Reverse: 5’ -AACATATTTGCATTGAGAAC-3’	55 ℃ SD	196
5	Forward: 5’ -CAAGACTATCCACTGTTAGG-3’		
	Reverse: 5’ -GGACAAACACGTAGATTTCAATAAC-3’	52℃ Standard	220
6	Forward: 5’ -GCTGCATAGTTCATGTCTGC-3’		
	Reverse: 5’ -CACAGGTGAAAATCAGACAG-3’	50 ℃ SD	194
7.1	Forward: 5’ -CTGCCTTTTAACTCTTCTGC-3’		
	Reverse: 5’ -GGCTGGAGGTAACTTTCCAC-3’	50 ℃ SD	247
7.2	Forward: 5’ -GGCCACAGGTAAAGATGTGG-3’		
	Reverse: 5’ -CAGGCGGCTGCACATGGTCC-3’	55 ℃ Standard	200
7.3	Forward: 5’ -GCCTCTGCTCAGGATGCAGG-3’		
	Reverse: 5’ -GCCCCGGCTGGGAAGGATTC-3’	50 ℃ SD	191

SD: step-down PCR

For SSCP assay[9,10], 3 μL PCR product, added with 4 μL denaturing buffer (95 % formamide, 10 mM NaOH, 0.25 % bromophenol blue and 0.25 % xylene cyanol), were kept at 95 ℃ for 5 min and then loaded 5 μL denatured mixture sample directly into each of sample wells of GeneGel Excel, 0.5 mm thin, pre-cast polyacrylamide ready-to-run gel for DNA electrophoresis (Amersham Pharmacia Biotech Inc.). Electrophoresis was done on GenePhor DNA separation system (Amersham Pharmacia Biotech Inc.) at 150 Voltage for 10 min and then 600 Voltage for 2 hours at 4 ℃. At the end of running, silver stained the gel with DNA silver staining kit (Amersham Pharmacia Biotech Inc.) in accordance with the manufacturer’ s protocol.

Expression and purification of GST fusion protein

The coding sequence of protein fragment for expression was cloned in frame into the *E.coli* GST fusion expression vector pGEX-4T2 (Amersham Pharmacia Biotech Inc.), then transfected the construct into *E.coli* BL21(DE3). The protein was expressed with IPTG induction for 3 hours and purified by glutathione-agarose (Sigma) column.

TRAP telomerase activity assay

HCC cells SMMC-7721were lysed in lysis buffer(10 mM TrisHCl pH7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 5 mM 2-mercaptoethanol, 0.5 % CHAPS, 10 % glycerol) on ice for 30 min and then centrifuged at high speed for 30 min, the suspension containing telomerase was used for TRAP assay. The GST-fused protein was incubated with cell extract for 10 min at 4 ℃ before subjecting to telomerase extension according reference[11]. Telomerase products were separated on 10 % polyacrylamide gels, which were stained with silver nitrate.

RESULTS

5 alterations were found in 12 tumor cell lines

We mainly screened the point mutations of LPTS gene in 56 liver cancer cell lines by means of SSCP. For chromosome 8p23 is a hot spot containing tumor suppressor in many types of tumors except for liver, including ovarian cancer and head and neck tumors, we checked LPTS gene in another 7 ovarian cancer cell lines and 7 head and neck tumor cell lines altogether. Among the cell lines analyzed, about half of those are homozygous in region between D8S550 and D8S518 on chromosome 8p23, covering the LPTS gene, by fine mapping of allele deletion (loss of heterozygosity) in Chromosome 8p.

After PCR amplification the fragment, PCR products were checked for specificity on 2 % agarose gel. Only the good specific amplification could be used in the latter assay (Figure 1). The specific PCR products were separated in PAGE gel and stained with silver nitrate. The abnormal band patterns, such as loss or gain of extra bands, different migration of bands, comparing to the negative and positive controls which had been sequenced in advance, suggested this sample might have point mutations (Figure 2). The products with abnormal migration pattern were sequenced and determined. The point mutations as shown in Table 2, were mainly located in exon 2, exon 5 and exon 7. In exon 2, there were two types of alterations, C (161) changed to T, predicted effect was the change from alanine to valine in cholangiocarcinoma cell CCLP1, and the other was C (183) to T, which was silent at the protein level and might therefore be considered as a polymorphism. In exon 5, only one point mutation of A (497) to G in HCC cell line PLCPRF5 was found, causing the encoded amino acid changing from tyrosine to cysteine. Two frequent mutations clustering at positions 778 and 880 were found in exon 7. The A778G changed the threonine residue to an alanine. All cell lines harboring this polymorphism were heterozygous at position 778 with the second allele retaining the wild type. The second mutation cluster A880T caused a change from serine to cysteine.

Table 2 The alterations of LPTS-S and LPTS-L in tumor cell lines

Cell line	Cell type	Mutation	Exon	Codon	Predicted effect
CCLP1	Cholangiocarcinoma	C161T	Exon 2	gct→gtt	Ala→Val
HepG2	HCC	A701A, G(LPTS-S)	Exon 7	Outside ORF	/
		A778A, G(LPTS-L)		aca→gca	Thr→Ala
HepT1	HCC	C183T	Exon 2	gcc→gct	Ala
MEK	HCC	A701A, G(LPTS-S)	Exon 7	Outside ORF	/
		A778A, G(LPTS-L)		aca→gca	Thr→Ala
SNU182	HCC	A701A, G(LPTS-S)	Exon 7	Outside ORF	/
		A778A,G(LPTS-L)		aca→gca	Thr→Ala
Li7A	HCC	A803T(LPTS-S)	Exon 7	Outside ORF	/
		A880T(LPTS-L)		agc→tgc	Ser→Cys
Li21	HCC	C183T	Exon 2	gcc→gct	Ala
		A803T(LPTS-S)	Exon 7	Outside ORF	/
		A880T(LPTS-L)		agc→tgc	Ser→Cys
MZCHA1	HCC	A803T(LPTS-S)	Exon 7	Outside ORF	/
		A880T(LPTS-L)		agc→tgc	Ser→Cys
PCI-SG231	HCC	G111C	Exon 1	Before ATG	/
		A803T(LPTS-S)	Exon 7	Outside ORF	/
		A880T(LPTS-L)		agc→tgc	Ser→Cys
PLCPRF5	HCC	A497G	Exon5	tat→tgt	Tyr→Cys
IGR-OV1	Ovarian tumor	A701A, G(LPTS-S)	Exon 7	Outside ORF	/
		A778A,G(LPTS-L)		aca→gca	Thr→Ala
SW579	Head and neck tumor	C183C, T	Exon 2	gcc→gct	Ala

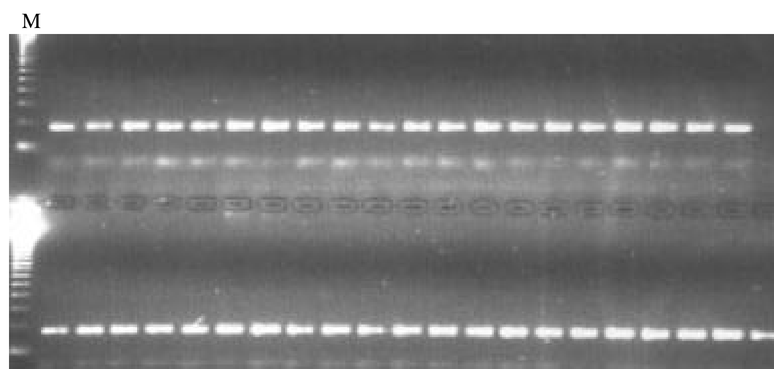

Figure 1 Agarose gel analysis of PCR products for SSCP assay. Agarose gel separation was used to determine the specificity of PCR reaction. PCR products with only one specific band of right size in the gel can be used to SSCP assay. M: 100 bp ladder.

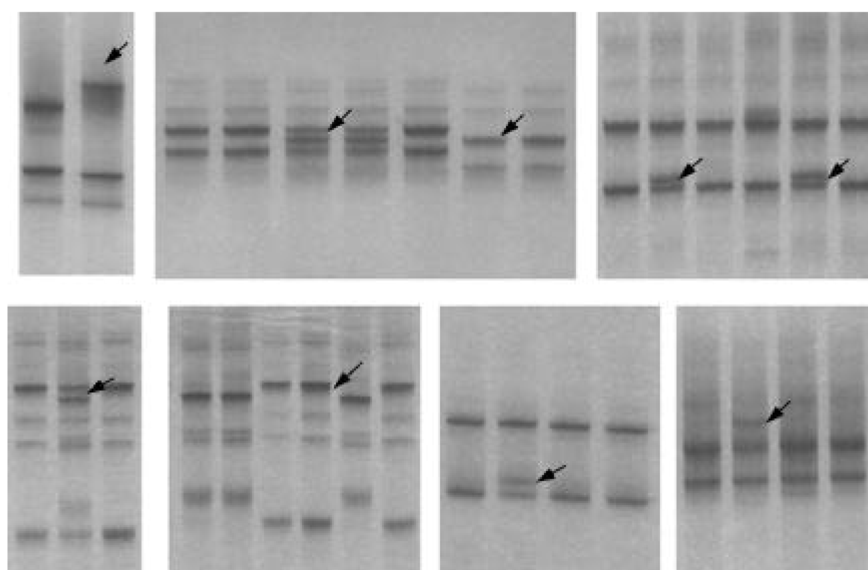

Figure 2 Some different electrophoresis patterns in SSCP assayof each exon. Arrows showed the differences, including extra bands, differential bands shifting, and so on.

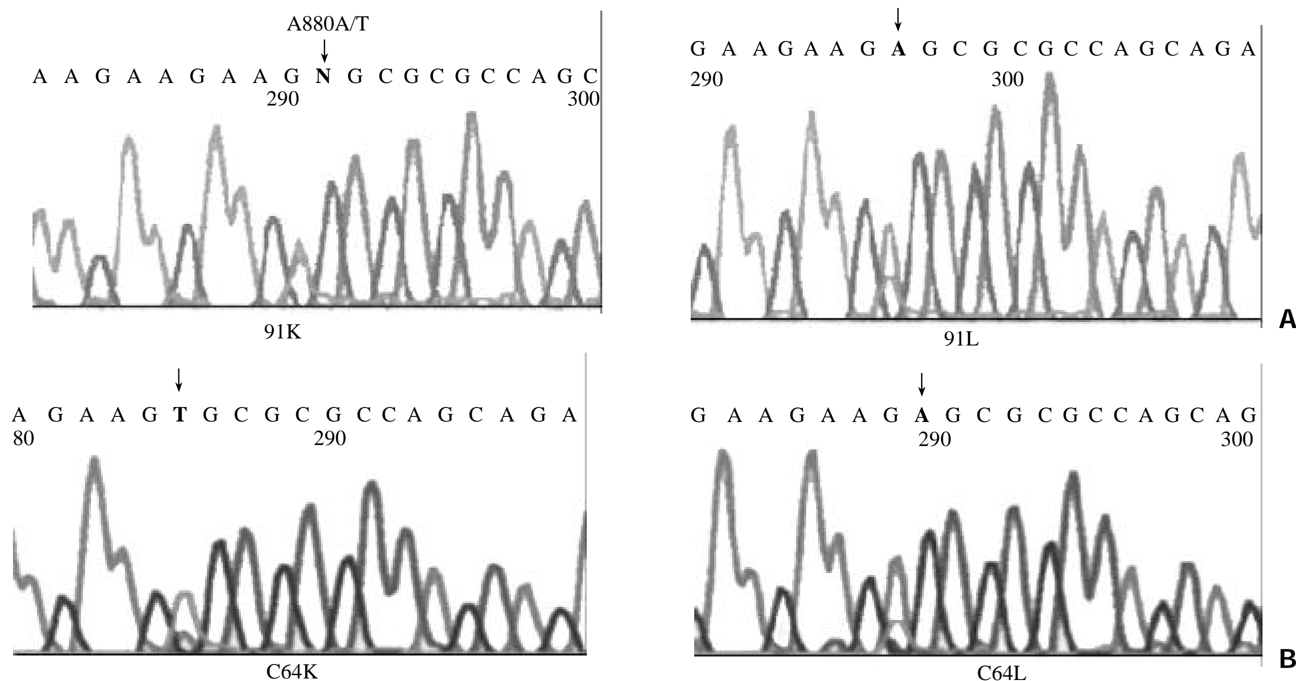

Figure 3 The sequences of mutation site of 880 in two pairs of HCC tissues. (A) HCC sample 91K, 91L; (B) HCC sample C64K, C64L. 'K' represents HCC tissue and 'L' represents adjacent nontumorous liver tissue. Arrow indicated the site of 880.

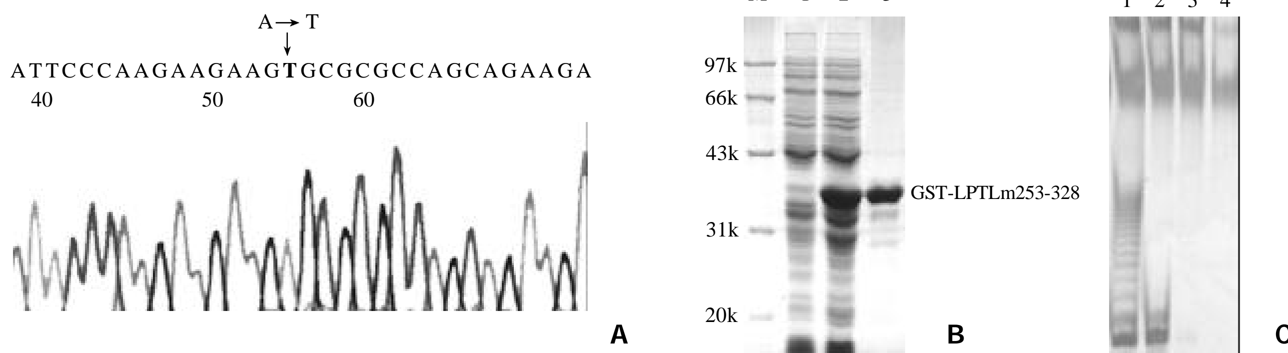

Figure 4 The point mutation in position 880 has no effect on the telomerase inhibitory activity of LPTS-L. A. The 'A' in position 880 of LPTS-L transcript was mutated to 'T'; B. The expression and purification of GST-LPTLm253-328, 1, without IPTG induction; 2, IPTG induction; 3, purified protein. C. The telomerase inhibiting activity assay in vitro of mutant protein LPTLm253-328 in SMMC-7721 cell extract. 1-4 were 0.1, 0.5, 1, 5 μg protein added in cell extract for 10 min, respectively.

Position (880) A to T was identified in 7 patients (10 %)

From the assay in HCC and other tumor cell lines, we can see that the major point mutations are in exon 7. The point mutation in 778 and 880 nt caused threonine and serine which being the candidate sites for phosphorylation changed to alanine and cysteine. The mutation in 880 nt turned to the cysteine, which might form disulfide bond and affect the conformation of the protein. It would be very important to confirm the existence of these two mutations in HCC tissues. Next, we collected 70 pairs of HCC samples, amplified the exon 7 and sequenced directly. In all 70 pairs samples, we could not find the point mutation in position 778, whereas the mutation in site of 880 was detected in 7 HCC samples and the mutation frequency was 10 %. The mutation in the site of 880 was also A/T heterozygous, which was shown double pits in sequence reaction (Figure 3).

The significance of A(880) mutation

LPTS-L/PinX1 possessing strong telomerase inhibitory activity had been deternmined by *in vivo* and *in vitro* experiments. To study the effect of telomerase inhibitory function of A(880) mutant, we designed PCR primer with point mutation at the site of 880, 5'-GAA TTC CCA AGA AGA AG*T* GCG CGC CA-3', the mutated site of 'T' was indicated in italic type. We subcloned the mutated cDNA fragment (from amino acid subunit 253 to 328) into GST fusion expression vector pGEX-4T2 and purified the GST-fused mutant protein. After TRAP telomerase activity assay, we found that the mutant protein still had strong telomerase inhibitory activity in HCC cell line SMMC-7721 cell extract (Figure 4). From these results, we concluded that the point mutation in position 880 of LPTS gene had no effect on telomerase inhibition.

DISCUSSION

Somatic inactivation of a tumor suppressor gene is usually achieved by intragenic mutation in one allele of the gene, with subsequent by loss of a chromosome region that spans the second allele, showed loss of heterozygosity (LOH). Among the various deleted chromosome regions identified by several laboratories, chromosome 8p has a particularly high frequency

of LOH[2, 12-14] and a growing body of evidence suggests that chromosome 8p is active in many types of carcinogenesis and metastasis including liver, breast, colorectal, prostate, lung, head and neck, pancreatic and urinary bladder carcinomas[14-18]. However, there have been no reports of the identification of a tumor suppressor gene on 8p.

LPTS gene is a possible candidate tumor suppressor localized in 8p23 region, which is a negative regulator in cell proliferation[3]. According to Kundson' s tumor suppressor "two-hit" model[19], one tumor suppressor needs to be hit twice to be inactivated. To clarify the relationship between LPTS gene and HCC, we searched for point mutations in all seven exons of LPTS gene in HCC tissue samples and HCC cell lines. In 70 tumor cell lines and 70 pairs of HCC tissues analyzed, there were mainly two point mutations in exon 7, position 778 and 880. The mutation at the site of 778 was found only in several HCC cell lines, but not in clinical HCC tissues. The mutation at the site of 880 has the mutation frequency of 10 % in HCC tissues, but the mutation has no effect of the telomerase inhibitory activity of LPTS-L/PinX1 protein, suggesting that this position is a polymorphism site of LPTS gene.

During our preparation of this manuscript, another research group in Korea reported their research work of genetic analysis of LPTS gene in HCC sample from South Korea. They also found that LPTS gene indeed had very high LOH frequency (34.5 %) in HCC patients, but also no point mutations could be found in HCC patients from South Korea, same with our results in Chinese HCC patients[20].Taken the above data together, LPTS gene has high frequency of LOH in HCC, but has no point mutation in the last allele, suggesting that inactivation of LPTS gene may not be caused by point mutation. However, we cannot rule out completely the epigenetic inactivation, such as methylation, acetylation etc[21-23]. Indeed, we found that there were two CpG islands, about 1.2 kb in length, in the upstream of LPTS gene transcription initiating site. Study of the methylation status in the regulation of LPTS gene is being performed now.

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